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14. ABSTRACT We selected Saccharomyces cerevisiae mutants that suppressed the G1 arrest and lethality observed following expressionof BRCA1 in yeast. This genetic screen identified conserved interactive components of the CCR4 damage responsenetwork that participate in mRNA elongation, transport and decay. These genes confer resistance to IR and UV as well astranscription elongation inhibitors. Since transcription elongation is regulated by phosphorylation of the RNA polymeraseII (RNAPII) carboxy terminal domain (CTD), we examined the status of RNAPII CTD phosphorylation following BRCA1induction. BRCA1-induced cleavage of the phospho-CTD was observed in WT, but not in mutant suppressor strains. Bothlethality and CTD cleavage was suppressed by cancer-related mutations in the BRCT domain of BRCA1 in WT yeast.Using co-immunoprecipitation, we determined that Spt4p and Dhh1p physically interact with BRCA1 in yeast, while theconserved human orthologs of Dhh1p (Ddx6p) and Spt5p interact with BRCA1 in human MCF7 cells following DNAdamage. Immunofluorescent colocalization of BRCA1, Spt5p and Ddx6p at cytoplasmic P-bodies in MCF7 cells suggeststhat BRCA1 shuttling plays a key role in mRNA decay. We hypothesize that defects in BRCA1-mediated RNAPII CTDcleavage and cytoplasmic mRNA shuttling following DNA damage are critical early events in the onset of breast cancer.

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Introduction

In 5-10% of breast cancers, inherited heterozygous defects in either the BRCA1 or BRCA2 genes have been associated with an enhanced risk of early onset cancer. These tumor suppressor genes normally function to maintain genome integrity by participating in the repair of DNA damage. The onset of these cancers is thought to occur following the rare loss of the wild type allele and subsequent environmental exposure of cells to physical or chemical agents that damage DNA. Due to the associated repair defects that occur following loss of the wild type BRCA1 allele, DNA lesions persist and destabilize the genome resulting in gross chromosomal rearrangements and/or aneuploidy. It is this severe DNA damage-induced genome destabilization that has been proposed to mediate cancer initiation and drive neoplastic progression.

Although inherited defects in BRCA1 or BRCA2 are clearly genetic predisposition factors for contracting breast cancer and their repair related functions have been extensively characterized, the precise underlying molecular mechanism responsible for the onset of breast cancer has remained obscure and enigmatic. This is due to the complex pleiotropic phenotypes associated with defects in the BRCA1 and BRCA2 gene products. These genes have been found to function in multiple pathways that maintain genomic integrity through their impact on the repair of a variety of genomic lesions including DNA double strand breaks (DSBs). BRCA1 is particularily complex as it has been found to participate in recombinational repair of DSBs, non-homologous end joining repair of DSBs, cell cycle checkpoint arrest, transcriptional regulatory mechanisms, chromatin remodeling as well as centrosome duplication [1 2 3 4]. Moreover, the protein product of BRCA1 has been found to physically interact with a bewildering array of co-factors in separate, multi-component repair complexes [5 6 7] that participate in a wide variety of DNA metabolic processes to maintain genome stability following DNA damage.

Although BRCA1 can be clearly described as “promiscuous” due to its multitude of physical interactions with proteins implicated in repair, the tumor suppressor activity is not widespread but surprisingly confined to that of the breast and ovary. Resolution of this enigma has been elusive and problematic. One hypothesis suggests that, BRCA1 interacts with a small subset of tissue specific cofactors in a single key pathway to mediate the tumor suppressive effects ascribed to BRCA1. Alternatively, rare defects among the many proteins found to interact with BRCA1 or defects in the expression of these proteins [8] may collectively contribute to the overall genetic risk of contracting breast or ovarian cancer. Support for the former hypothesis can be inferred from the relatively few breast cancers found to contain mutations in recently identified repair related proteins that physically interact with BRCA1. Since defects in these proteins confer a genetic risk of contracting breast cancer at levels much lower than the 5-10% of breast cancers found to contain defects in BRCA1 or BRCA2 [9], this suggests that BRCA1 functions in many pathways unrelated to tumorgenesis. Moreover, it further suggests that additional unidentified gene products in a single key pathway essential for tumorgenesis may be responsible for the tissue specific suppressor effects of BRCA1.

Establishing the normal molecular functions of BRCA1 in useful mammalian model systems has been difficult due to the absolute requirement of wild type BRCA1 in normal embryogenesis [10]. Furthermore, overexpression of BRCA1 in mammalian cells is

toxic [11] and the protein product unstable as it is prone to degradation in a variety of pathways [12 13 14]. Subcellular localization of BRCA1 has also been controversial. Although the vast majority of functional studies have localized BRCA1 activity to within the nucleus, a few studies have described BRCA1 as a shuttling protein [15 16 17] with unknown functions located in the cytoplasm. As an alternative approach for studying this problematic repair protein, we have utilized the yeast *Saccharomyces cerevisiae,* which has served as the preeminent model organism for the identification of the genetic controls associated with DNA repair and checkpoint functions in eukaryotes. This organism also serves as an important model for the elucidation of complex human systems since it shares a high level of genetic and functional homology with humans [18]. In fact most of the gene products involved in the repair of DSBs in humans were first identified in yeast.

Yeast appears to be an excellent model organism for elucidating BRCA1 function since heterologous expression of BRCA1 causes slow growth and lethality in both haploid [19] and diploid [20] repair proficient wild type (WT) yeast. Furthermore, expression of the BRCA1 C terminal domain (BRCT) can stimulate transcriptional transactivation in yeast [21] as it does in human cells. Mutations within the two tandem C terminal BRCT domain repeats of BRCA1 that have been associated with breast cancer disease abrogate this slow growth phenotype [19] as well as the transactivation potential suggesting that transcription related effects of the BRCT domain may be important to tumorgenesis as well as BRCA1-induced yeast lethality. Moreover, the BRCT domain appears to be a common feature of proteins involved in checkpoint and DNA repair related functions [22 23] since it mediates protein-protein interactions in yeast [24] and human [25] cells. Polymorphisms within BRCA1 not associated with breast cancer do not interfere with the slow growth phenotype in yeast. Taken together, these results suggest that these phenotypes in yeast accurately reflect biologically relevant molecular functions of BRCA1 in human cells.

In order to determine a fundamental basic role that may underlie the tumorgenic potential of BRCA1 in breast cancer, we utilized the vast evolutionary distance between humans and yeast to identify highly conserved evolutionarily "invariant" proteins that still maintain functional interaction with BRCA1 in yeast. The rationale was to use yeast as an evolutionary "filter" to identify those conserved proteins that, through severe functional constraints, have maintained the ability to interact with BRCA1 over a large evolutionary distance. We hypothesize that these proteins will perform a highly conserved function in yeast that is resistant to random mutational drift and may identify the biological process critical to cancer initiation and progression. We previously described that a prolonged G1 arrest and lethality occurs following heterologous expression of BRCA1 in diploid yeast [20]. Using yeast as a model system to identify new BRCA1 interactive gene targets, we found in a screen of isogenic ionizing radiation (IR) sensitive deletion mutants [26] that null deletions of the IR resistance genes *CCR4* or *DHH1* suppressed BRCA1-induced G1 arrest and lethality [20]. We proposed that BRCA1 interacted with these proteins in a large complex to interfere with their normal transcription related functions essential for yeast cell survival. In this report we have extended this approach by screening in addition to IR sensitive mutants, a random unselected pool of yeast deletion mutants to identify suppressors of the G1 arrest and lethality. Using this combined screening approach we identified a large number of genes implicated in processes related to mRNA metabolism. Specifically we identified genes

that participate in mRNA transcription elongation as well as mRNA export and decay. Among these genes we identified the highly conserved *spt4Δ* as the most potent genetic suppressor of BRCA1-induced G1 arrest and lethality in yeast. Carboxy (C) terminal truncations of the cognate Spt5 protein, which physically interacts with Spt4p also suppressed BRCA1-induced lethality suggesting an important role of the conserved human DSIF complex (Spt4-Spt5) in the BRCA1 tumorgenic response. In support of this hypothesis, yeast and human Spt4 proteins were found to physically interact with BRCA1 in yeast while human Spt5p was found to physically interact with Spt4p and BRCA1 in human cells. Furthermore, we show that similar to interactions observed in human cells, BRCA1 genetically and physically interacts with the phosphorylated C terminal domain (CTD) of RNA polymerase II in yeast. Moreover, expression of BRCA1 results in RNA polymerase II CTD cleavage that is abrogated by cancer related defects in the BRCT domain of BRCA1. Other identified suppressors of BRCA1 induced lethality link transcription elongation to mRNA export and decay suggesting a model in which BRCA1 interacts with RNA polymerase II at DNA damage sites to mediate cleavage and release of the arrested transcription complex from the DNA template followed by transport to the cytoplasm for mRNA degradation. Due to the highly conserved nature of this process, we propose that defects in this damage signaling response may be critical for the initiation of breast cancer.

Body:

Task 2. For yeast genes with human orthologs, we will determine if physical protein-protein interactions occur between the newly identified proteins and BRCA1 in yeast and human cells. (Months 13-30).

a. Design and construct a series of plasmids for yeast two hybrid analysis using BRCA1 as the "bait" and candidate yeast (or human orthologs) identified in Task 1 as the "prey". (Months 8-30).
b. For candidate genes that show physical interaction in yeast, design and construct a series of plasmids for mammalian two hybrid analysis using BRCA1 as the "bait" and candidate human genes as the "prey". (Months 12-36).
c. Confirm by co immunoprecipitation techniques the occurrence of physical interaction of BRCA1 protein with candidate human proteins that were previously shown positive for two hybrid interactions in mammalian cells. (Months 16-36).

As discussed in the year 2 report, significant two-hybrid interactions were not seen for two of the main interactive targets identified in yeast (Spt4p and Dhh1p). We failed to see any significant two-hybrid interaction among these proteins or their human orthologs, however, we were able to successfully co-immunoprecipitate (co-IP) BRCA1 and Dhh1p in yeast cells as well as co-IP DDX6 and BRCA1 proteins expressed in human MCF7 cells. As described in our year 2 report, we have successfully identified using co-IP techniques, interactions of yeast Spt4p with BRCA1 using anti Spt4p as the

precipitating antibody in the co-IP reaction. However, using this same antibody to detect endogenous levels of Spt4p in cells that are expressing BRCA1 has been problematic as very small amounts of Spt4p can be detected following either western blot analysis of whole cell extracts or following IP reactions using anti-BRCA1 antibody as the co-IP reagent. We therefore overexpressed a V5 tagged Spt4p fused to the GAL1 promoter in pDEST52 (*GAL::*SPT4-V5) concomitantly with BRCA1 in WT yeast and performed IP reactions using either anti-BRCA1 antibody (AB-1) or normal mouse immunoglobulin (Fig 1). Two technical details have allowed us to successfully optimize the detection of BRCA1 specific interactions with Spt4p in yeast. We varied the NP40 concentration from 0.5-1.0% for each whole cell extract and cell extracts were precleared for 12-18 hours

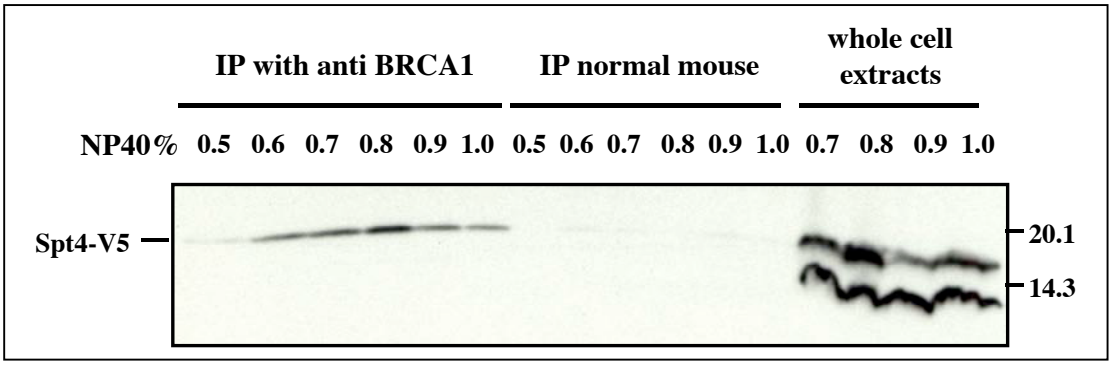

Fig 1. Spt4p interacts physically with BRCA1 following co-expression in yeast. WT yeast cells (BY4743) were transformed with selectable plasmids containing GAL::BRCA1 and GAL::Spt5-V5 fusion constructs. Following growth under repressive conditions in glucose, cells were washed in water and transferred to galactose and BRCA1 and Spt4p proteins were induced for 16 hours at 30oC. Whole cell extracts were made by bead beating cells resuspended in lysis buffer at various NP40 concentrations (plus protease inhibitors) and preadsorbed with 3 ug of normal mouse IgG and an equal volume of agarose A + G beads. Following preadsorption, the beads were spun down (3500x) and the supernate equally divide for IP reactions (12-18 hours at 4oC) with either 3 ug of anti BRCA1 antibody or normal mouse IgG plus 40 ul of agarose A + G beads. Beads were spun down and washed 2x in 300 ul of extraction buffer. Protein was eluted from the beads at 100oC and run on a gradient 4-15% PAGE gel (Criterion, Biorad). Aliquots (2200 ug total protein) of the whole cell extracts using 0.7 to 1.0% NP40 are indicated. Seperated proteins were electroblotted to nitrocellulose and probed with anti V5 antibody (Invitrogen). Protein visualization was performed using an ECL kit (Amersham). Maximal interaction between BRCA1 and Spt4p was observed at an NP40 concentration of 0.8%. Spt4p detected from whole cell extracts typically separate as a doublet Interaction of BRCA1 and Spt4p always was observed with the higher molecular weight form of Spt4p (ca 15 kDa). This may represent a hyperphosphorylated form of the protein.

with 3 ug of normal mouse IgG prior to immunoprecipitation. When these proteins are concomitantly expressed in WT yeast, maximal co-IP interaction between BRCA1 and Spt4p was observed at 0.8% NP40 concentration and no interaction was observed using normal mouse IgG as the precipitating antibody in the co-IP reaction (Fig 1). Similarly in human MCF7 cells, maximal immunoprecipitation of BRCA1 with AB-1 antibody occurred following extractions in 0.8% NP40 (data not shown). Therefore we have confirmed that a physical interaction occurs between Spt4p and BRCA1 in yeast.

Since no significant two hybrid interactions were observed in yeast for two of the major suppressors of BRCA1-induced lethality (Spt4p and Dhh1p) or their human counterparts (Spt4p and DDX6), we postulate that these proteins are interacting in yeast and human cells indirectly as components of a large multicomponent complex. We

therefore chose not to pursue further two hybrid analysis studies in human cells (Task 2 b above) and instead have concentrated our efforts on confirming by co-IP (which can detect indirect physical interactions of proteins within multicomponent complexes) interaction of candidate human proteins with BRCA1. We therefore examined whether Spt5p (the cognate interactive partner of Spt4p and for which an excellent monoclonal antibody is commercially available) interacts with BRCA1 in human cells. This protein has been found by us (in MCF7 cells; data not shown) or by others to be physically associated together as members of the DSIF (DRB sensitivity-inducing factor) transcription elongation complex. DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) is a transcription elongation inhibitor [27]. These DSIF complex proteins regulate transcription elongation by physically binding to RNA polymerase II (RNAPII). In a complex pTEFb dependent regulatory process, phosphorylation events on the long RNAPII heptapeptide C terminal repeat domain

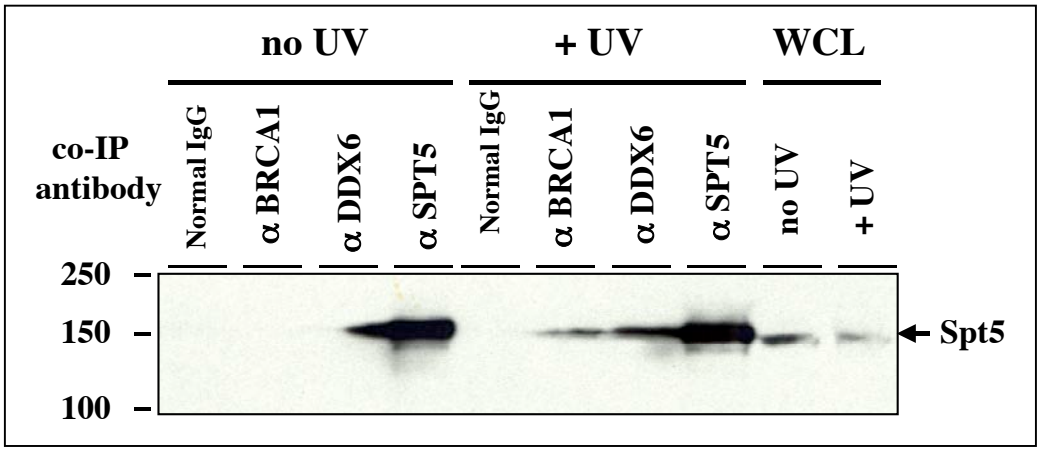

Fig 2. Spt5p interacts with BRCA1 and Ddx6p following UV irradiation in human MCF7 cells. MCF7 cells were grown to confluency in 100mm dishes and either left unirradiated or exposed to 20 J/m2 of ultraviolet (UV) light. Following UV, cells were reincubated at 37oC with fresh medium. After 2 hours cells were harvested by washing 3x in sterile ice cold PBS followed by cell lysis in 1 ml of extraction buffer for 1 hour with slow shaking at 4oC. Co-immunoprecipitation conditions, protein separation and visualization by western blotting was the same as that described above in Figure 1. Whole cell lysates (WCL) represent 200 ug of total proteinInteraction of Spt5p with BRCA1 and Ddx6p was only observed following UV irradiation.

(CTD) as well as on DSIF are modulated to facilitate transcription elongation [28]. Hyperphosphorylation of the RNAPII CTD is associated with elongating transcription complexes, while hypophosphorylation of the CTD has been associated with transcription initiation at or near the promoter site [29]. We have speculated that BRCA1 may only interact with these proteins following transcription arrest due to physical blockage of the elongating transcription complexes by DNA lesions in the transcribing strand. To test this we examined whether Spt5p and DDX6 interact with BRCA1 in human MCF7 cells that were either unirradiated or UV irradiated with 20 J/m^2 two hours prior to protein extraction by cell lysis (Fig 2). In this experiment, we observed significant co-IP interaction of BRCA1 with Spt5p as well as Ddx6p with Spt5p following UV irradiation. Little or no co-IP interaction was observed for Spt5p with BRCA1 or Spt5p with Ddx6p in unirradiated MCF7 cells. These results suggest that BRCA1 interacts with Spt5p and

Ddx6p in a DNA damage dependent manner and are consistent with a model in which elongating transcription complexes arrested by DNA damage interact directly with BRCA1.

Many BRCA1 protein-protein interactions are dependent on the BRCA1 Carboxy terminal domain (BRCT) [30]. We therefore examined whether physical interaction of Ddx6p with BRCA1 was dependent on the BRCT domain. To do this we compared protein-protein interactions between BRCA1 and Ddx6p in MCF7 cells that express full length BRCA1 and HCC1937 cells that express a mutated BRCA1 which is

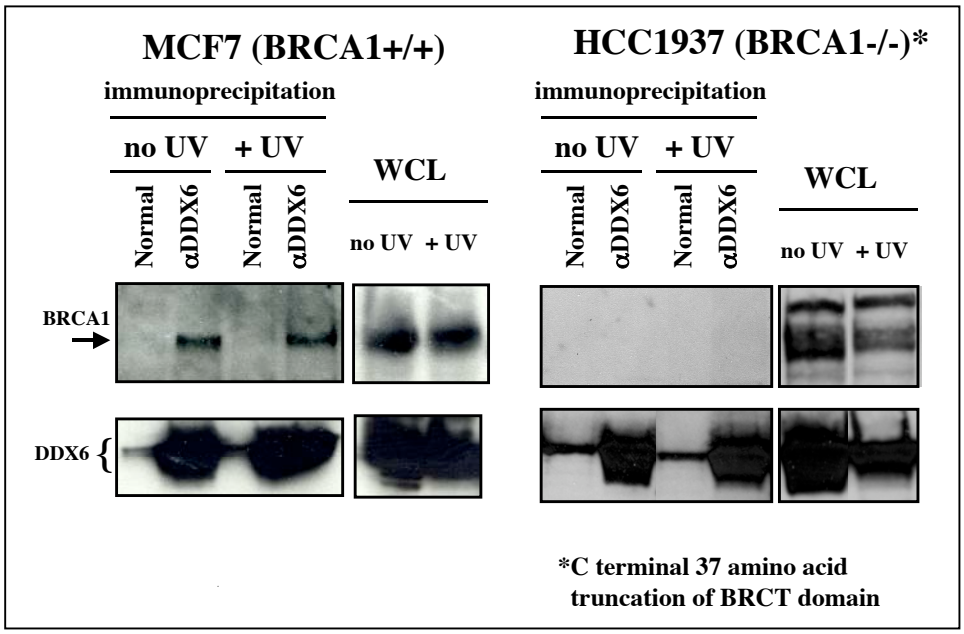

Fig. 3. Physical interaction between BRCA1 and DDX6 requires a functional BRCT domain in BRCA1. Human MCF7 (expressing full length BRCA1) and HCC1937 (expressing a C terminal BRCA1 truncation) cells were grown, UV irradiated and lysed as described in Figure 2. Preclearing, co-immunoprecipitations and protein detection of BRCA1 and DDX6 by western blot analysis were identical to that described in Figure 2. Whole cell lysates (WCL) were all prepared in 0.8% NP40 extraction buffer. Normal = normal mouse IgG. Ddx6p runs as a 54 kDa protein. Ddx6p interacts with BRCA1 in both unirradiated and UV irradiated MCF7 cells. No physical interaction between BRCA1 and Ddx6p could be detected in extracts prepared from HCC1937 cells.

truncated (by 34 amino acids) at the carboxy terminus thereby rendering the BRCT domain of BRCA1 non-functional [31]. We prepared whole cell extracts from unirradiated and UV irradiated MCF7 cells (which contain a functional full length BRCA1 protein) that produce high endogenous levels of BRCA1 and Ddx6p. Using co-IP analysis of protein-protein interaction, we observed significant interaction between BRCA1 and Ddx6p in both unirradiated and UV-preirradiated MCF7 cells (Fig 3). However, in HCC1937 cells we failed to find any significant interaction with the truncated form of BRCA1 following successful immunoprecipitation of large quantities of Ddx6p. These results clearly indicate that BRCA1-Ddx6p interaction is dependent on a functional BRCT domain.

BRCA1 is required for resistance to the DNA damaging agent methyl methane sulphonate (MMS) which causes alkylating damage that results in the accumulation of DNA double strand breaks [32]. In an attempt to induce a constant level of exposure to a

DNA damaging agent (rather than a single pulse of UV irradiation), we exposed MCF7 to 0.1% MMS for 2 hours to examine the interaction of BRCA1 with Spt4p and Ddx6p in a manner similar to that as described in Fig. 2. Surprisingly, at this level of MMS exposure, BRCA1 and Spt5p proteins were completely degraded while Ddx6p was maintained at a constant level (data not shown). We therefore exposed MCF7 cells to a variety of concentrations of MMS (0.025 – 0.1%) for 1 or 2 hours followed by Western blot analysis to examine the persistence of BRCA1, Spt5p and Ddx6p at the protein level (Fig. 4). Interestingly, BRCA1 and Spt5p were degraded in a dose and time dependent manner, while Ddx6p protein levels were unaffected. These results

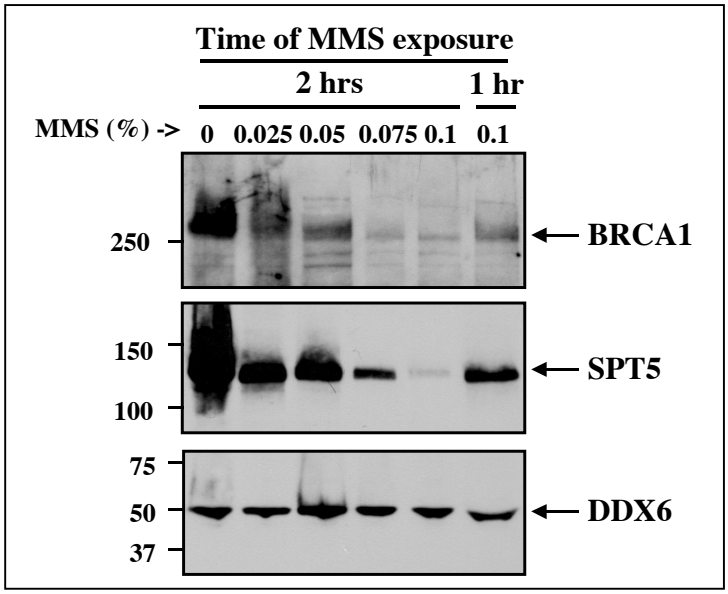

Fig. 4. BRCA1 and Spt5p are rapidly degraded following exposure of MCF7 cells to MMS. MCF7 cells were exposed to MMS at the indicated concentrations for 1 or 2 hours in complete RPMI medium. Whole cell lysates were prepared as described for Figures 2 and 3. Protein extracts were separated by PAGE and electroblotted to nitrocellulose. Proteins were detected with the appropriate antibody probes and visualized by western blotting as described for Figures 2 and 3. Each lane represents 200 ug of total protein. BRCA1 and Spt5p but not Ddx6p are rapidly degraded following MMS induced damage.

suggest that BRCA1 and the transcriptional elongation regulator Spt5p may be degraded together as interactive components of the same complex following MMS damage. Furthermore, the fact that Ddx6p is not degraded suggests that Ddx6p interactions with BRCA1 and Spt5p may be independent of those required for transcription elongation mediated by RNAPII.

BRCA1 interacts physically with RNAPII in mammalian cells and the DSIF complex (Spt4p-Spt5p) is known to interact with RNAPII to modulate CTD phosphoylation and transcription elongation. We examined the status of RNAPII following BRCA1 expression in yeast using an affinity purified polyclonal antibody raised against a synthetic phosphorylated heptapeptide repeat that was identical to that in the RNAPII CTD of yeast and human cells. Since the highly conserved transcriptional cofactor Spt5p is also known to interact with phosphorylated forms of the RNAPII CTD in yeast as well as human systems to regulate transcription elongation [33 34 35], we examined the status of RNAPII CTD phosphorylation following BRCA1 expression in WT yeast. The yeast and human RNAPII CTD is highly conserved and both share a repeated heptapeptide sequence (YSPTSPS) that can be variably phosphorylated at the second, fifth and seventh serine (S) residues [36].

In collaboration with Arno Greenleaf (Duke University) we have obtained an affinity purified antibody raised against a synthetic CTD peptide phosphorylated at the second and fifth serine [37]. Since hyperphosphorylation of the CTD is usually associated

with elongating mRNA complexes while hypophosphorylated forms of the CTD are associated with preinitiation complexes at the promoter, we examined whole cell extracts obtained from WT diploid yeast in which the *GAL::*BRCA1 fusion construct was expressed from a selectable high copy plasmid at various times. Cells were induced in synthetic complete galactose (GAL) containing medium lacking uracil (URA) to maintain selection for the plasmid. Whole cell extracts were prepared on dry ice in 95% ethanol to minimize protein degradation that often accompanies extractions performed on "wet" ice in 0.8% NP40 buffer with protease inhibitors. Protein containing lysates were separated by standard polyacrylamide gel electrophoresis (Criterion, Biorad) and electroblotted to nitrocellulose membranes. The membranes were subsequently probed with anti-BRCA1 (AB-1, Calbiochem) and anti-phospho-CTD antibody.

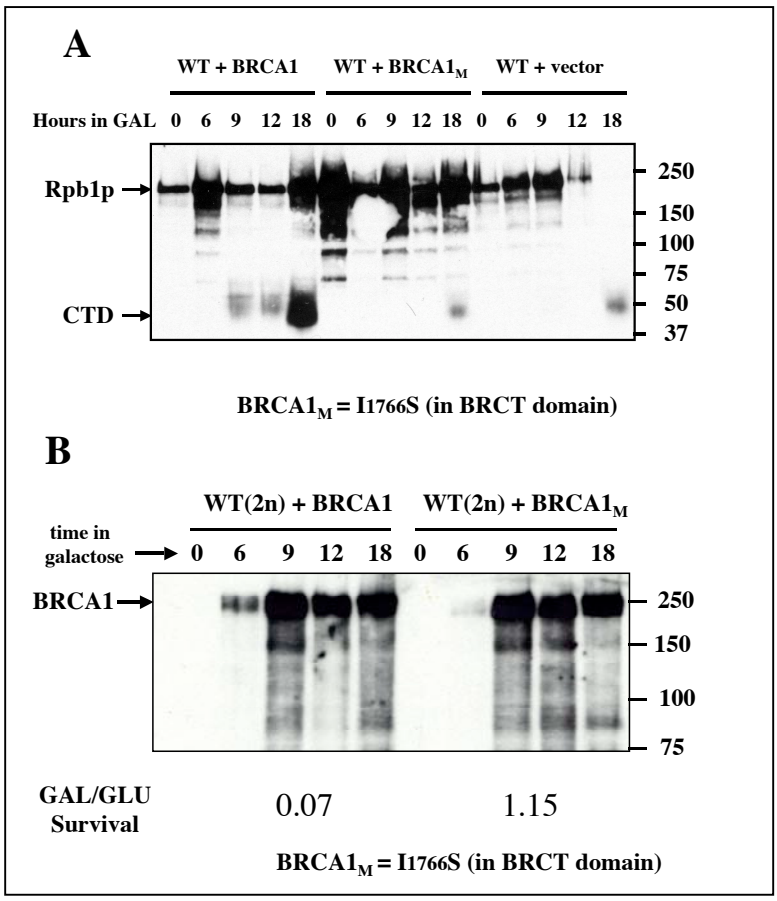

Fig 5. BRCA1 induces cleavage of a phosphorylated form of the RNAPII CTD. WT diploid (2n) yeast cells containing high copy plasmids bearing *GAL::*BRCA1 or *GAL::*BRCA1$_M$ fusion products or empty vector were induced in galactose (GAL) for the times induicated. Cells were extracted in ethanol on dry ice by bead bearting followed by speedvac evaporation. Residual dried protein pellets were separated by PAGE and electroblotted to nitrocellulose. Phosphorylated Rpb1p (Panel A) and BRCA1 (Panel B) were visualited by Western blotting using the appropriate antibodies as described in Figure 2 and text. The BRCA1$_M$ contains a breast cancer implicated mutation within the BRCT domain and substitutes a single amino acid at position 1766 (I to S). Expression of the BRCA1M within WT cells is non-lethal resulting in a survival fraction for colony forming ability on galactose containing plates vs. glucose containing plates (GAL/GLU) of 1.15. Expression of the WT but not the mutated BRCA1 results in cleavage of the RNAPII CTD.

Following Western blotting with the polyclonal anti-phospho-CTD antibody, we observed a novel 50 kDa cleavage fragment of the phosphorylated CTD released from the major 191 kDa catalytic subunit of RNAPII (*i.e.* Rpb1p) following BRCA1 expression in WT yeast (Fig. 5A). Cleavage of this heavily phosphorylated CTD fragment has not been previously described in yeast or human cells and was not observed following identical expression of BRCA1 protein containing cancer related mutations within the BRCT domain in WT yeast (Fig. 5A) or following expression of full-length non-mutated BRCA1 in mutant yeast strains *(def1Δ, ccr4Δ* or *dhh1Δ*) that suppressed BRCA1-induced lethality (data not shown). Expression of the mutated BRCA1 was similar to that

of the non mutated BRCA1 but completely suppressed lethality in WT diploid yeast (Fig 5B). The failure to demonstrate enhanced CTD cleavage following expression of the mutated form of BRCA1suggests that cleavage of the RNAPII may be the lethal mechanism following heterologous expression of BRCA1 in yeast. Furthermore, this CTD fragment was also induced in a time course dependent manner following UV irradiation of WT but not *ccr4Δ* yeast (data not shown) suggesting that UV mediated RNAPII cleavage is *CCR4*-dependent. Since Ccr4p appears to be a checkpoint protein [38], this suggests that CTD cleavage may be a DNA damage checkpoint signal mechanism in yeast and possibly human cells. These results suggest that BRCA1 mediated cleavage of a highly phosphorylated form of the RNAPII CTD may occur when transcription elongation has been "stalled" by DNA damaging lesions within actively transcribing genes to activate damage dependent checkpoint arrest in G1. This process (CTD cleavage) may occur prior to previously described ubiquitinylation-mediated degradation of stalled RNAPII complexes following DNA damage in yeast and human cells [36 39].

Given the identification of numerous transcription elongation regulators as suppressors of BRCA1 induced lethality including the Spt4p-Spt5p complex, we examined whether individual deletions of two nonessential genes that code for the regulatory kinase *CTK1* and the cyclin *BUR2* could affect the lethality following BRCA1 expression in yeast. Both Ctk1p and Bur2p (in concert with Sgv1p) have been found to specifically phosphorylate the RNAPII CTD to regulate transcription elongation [40 41]. Defects in either of these kinase activities has been previously associated with sensitivity to DNA damaging agents [26 42]. Deletion of *CTK1* resulted in a complete suppression of BRCA1 induced lethality following three days growth at 30ºC (Survival of relative colony forming ability on GAL *vs*. GLU = 0.92). However, deletion of *BUR2,* which slows the growth of the host strain, only partially suppressed BRCA1 induced lethality after five to seven days growth on selective synthetic complete medium (Relative survival of colony forming ability on GAL *vs* GLU = 0.31). The complete suppression of BRCA1-induced lethality by *ctk1Δ* suggests that phosphorylation of the repeated serine residues within the RNAPII CTD (*i.e*. at positions two and five) are critical to mediate BRCA1-induced lethality. It further indicates that the lethal effects of BRCA1 expression in yeast are through direct interactions with the RNAPII CTD to affect transcription elongation.

Task 3. Determine if newly identified BRCA1-interacting genes contribute to genomic stability following IR damage in human cells. (Months 13-36).

a. Using siRNA technology, down regulate the expression of BRCA1 in normal human fibroblast cells and determine if cells are sensitized to the killing effects of IR. (Months 13-18).
b. Using siRNA technology down regulate highly conserved, candidate BRCA1-interacting genes in normal human fibroblasts to demonstrate sensitivity to the killing effects of IR. (Months 16-36).

As described in the year 2 report, we have successfully used siRNA transfection techniques to down regulate BRCA1 expression in MCF7 human breast cancer cells that express BRCA1 as well as in immortalized normal (DU99) human cells (data not shown).

MCF7 cells were either transiently transfected with siRNA designed for the down regulation of BRCA1 mRNA or nonsense siRNA as a negative control. Furthermore, using a previously described plasmid based recombination system, we have found a 4.2 fold decrease in recombination frequency following BRCA1 ablation by RNA interference. Thus defects in the BRCA1 recombinational repair pathway can be readily assayed using this system.

Given the high degree of identity between the DEAD box helicase Dhh1p and its human ortholog Ddx6p (66% identical), as well as the ability of Ddx6p to partially suppress the ionizing radiation sensitivity of a *dhh1Δ* strain [20], we chose to examine the radiobiological effects of this protein in greater detail in human cells. Furthermore, abnormal enhanced expression of this protein has been implicated in both colorectal cancers [43] and hepatocarcinoma [44] suggesting it may have a regulatory role in the initiation of breast cancer as well. Finally, we have shown that *DHH1* functions as a checkpoint adaptation gene in response to ionizing radiation. It appears to be (like *CCR4*) a member of the RAD9 epistasis group of checkpoint repair proteins. Following deletion of *DHH1*, cells have a prolonged G1 to S phase transition following IR as well as defects in S phase in response to the S phase specific agents HU and MMS.

Recently, Dhh1p and DDX6 (as well as Ccr4p) have been shown to be obligate members of cytoplasmic processing (P) bodies which are the cellular sites for mRNA decay [45 46]. Our finding that deletion of *DHH1* and *CCR4* and not other key members of the CCR4-NOT complex suppress BRCA1 induced lethality suggests that it is the unique P-body related functions of these two

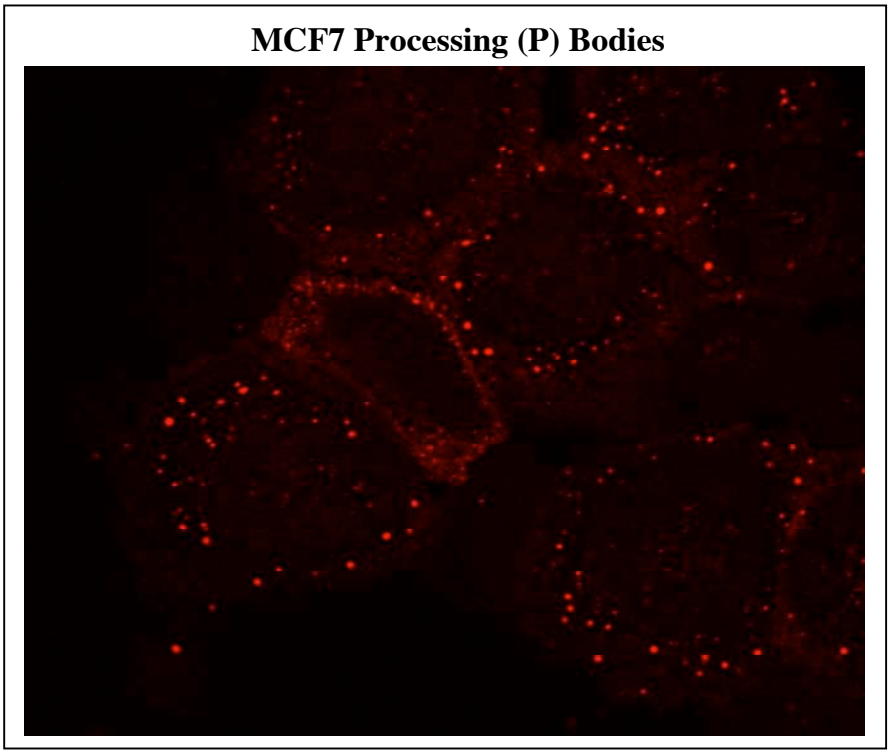

Fig. 6 MCF7 cells possess cytoplasmic processing bodies. MCF7 cells were seeded at 8 X 10^5 cells per 60 mm dish. 5 X 10^4 cells were placed on glass slides using a cytospin centrifuge adaptor and spun for 5 min at 600 rpm. Cells affixed to the slide were air dried. After fixing in 100% methanol, the cells were hybridized with rabbit anti-DDX6 (MBL) and detected with a rabbit secondary antibody that was conjugated with Texas Red. The photomicroscopic images were taken at 63X with a Leica confocal microscope.

genes that is responsible for suppression of BRCA1-induced lethality in yeast. Also, since DDX6 physically interacts with BRCA1 but is not degraded along with BRCA1 and

Spt5p in response to MMS treatment of MCF7 cells (Fig. 4), this suggests that DDX6 may play a role downstream of transcription arrest checkpoint signaling mediated by BRCA1 and DNA damage induced RNAPII complex elongation arrest. We hypothesize that following RNAPII transcription arrest by DNA damage, BRCA1, in the presence of the DSIF complex mediates RNAPII CTD cleavage releasing the mRNA and RNAPII complex from the DNA template strand to allow access of repair enzymes to the DNA damage site. BRCA1 interaction with the RNAPII CTD requires site-specific phosphorylation of the CTD. BRCA1 then "shuttles" the prematurely terminated mRNA RNAPII complex through the nuclear pore into the cytoplasm where it interacts with the cytoplasmic P bodies to initiate mRNA decay and proteosome degradation of the cleaved RNAPII and associated co-factors such as DSIF.

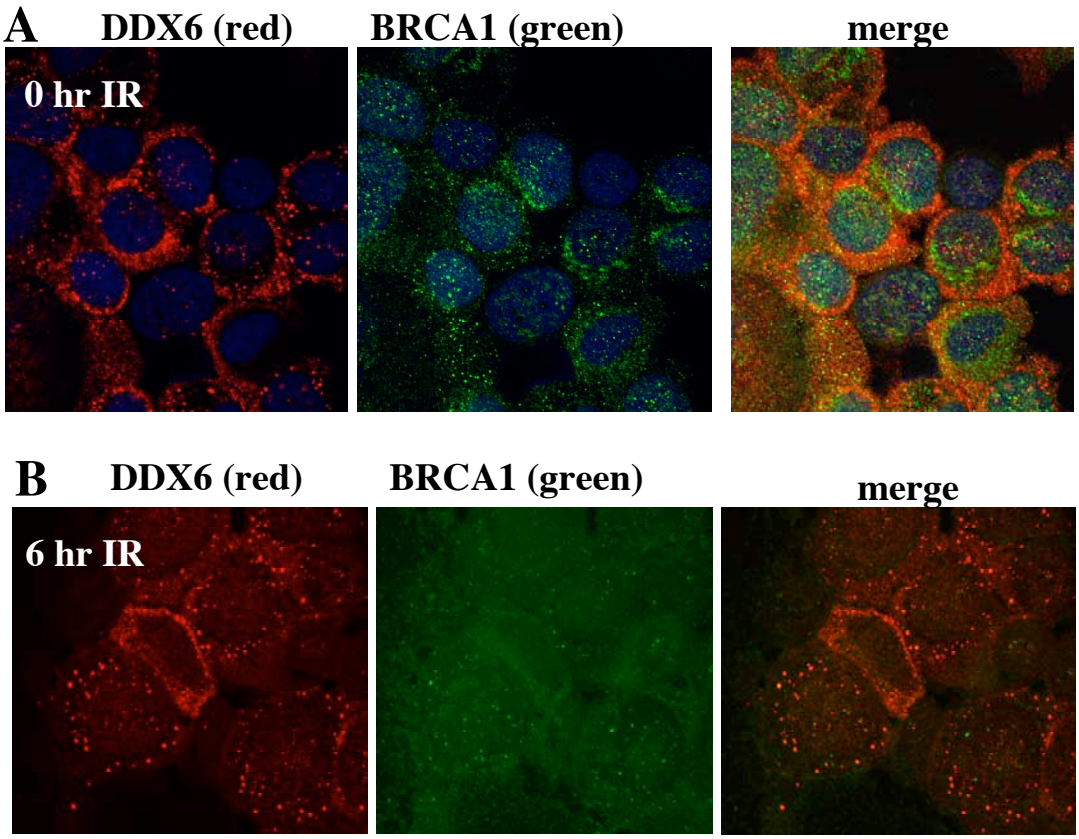

Fig. 7 DDX6 and BRCA1 colocalize to processing bodies in MCF 7 cells. MCF7 cells were seeded at 8 X 10^5 cells per 60 mm dish. . The cells were either unirradiated (Panel A) or irradiated with 5 Gy of ionizing radiation and incubated 6 hours before processing (Panel B). 5 X 10^4 cells were placed on glass slides using a cytospin adaptor. After fixing in 100% methanol, the cells were hybridized with rabbit anti-DDX6 (MBL) and visualized with rabbit secondary antibody conjugated with Texas Red. The cells were then hybridized with a mouse anti-BRCA1 (Oncogene) and detected with a mouse secondary Alexa Fluor 488 (green). The photomicroscopic images were taken at 63X with a Leica confocal microscope. The arrows on the enlarged merged image indicate colocalization of BRCA1 and DDX6

To gather experimental support for the above hypothesis, we sought to determine if BRCA1 was shuttled as a component of the transcription elongation complex to cytoplasmic P-bodies in a dose and time dependent manner. We therefore examined unirradiated and irradiated (UV or IR) MCF7 cells using indirect *in situ* immunofluorescence to determine if BRCA1 could be visualized in association with DDX6 at the cytoplasmic P-bodies following DNA damage. These were the same cells with which BRCA1 interactions could be identified with Spt5p and Ddx6p (Fig. 2). To establish a baseline, we first examined unirradiated MCF7 cells to determine if cytoplasmic P-body structures could be readily visualized using an anti-DDX6 antibody in undamaged cell that had been fixed on slides. Using a polyclonal rabbit anti-DDX6 antibody, MCF7 cells were fixed and hybridized with DDX6 specific antibody. Using a Texas Red conjugated secondary anti-rabbit antibody, numerous (>20) discrete P-bodies could be easily observed in the cytoplasm of the unirradiated MCF7 cells using confocal fluorescent microscopy (Fig. 6). Little or no DDX6 could be visualized in the nucleus.

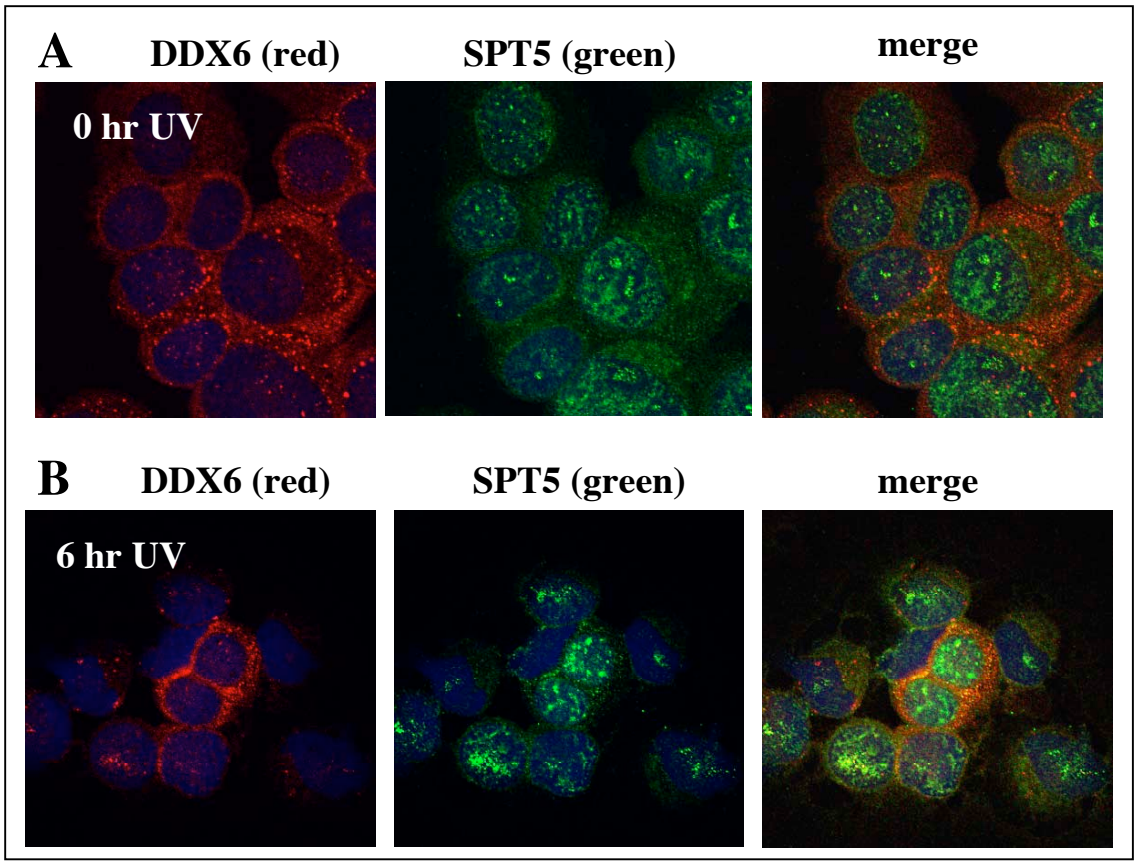

Fig. 8 DDX6 and SPT5 colocalize to processing bodies in MCF7 cells following DNA damage. MCF7 cells were seeded at 8 X 10^5 cells per 60 mm dish. The cells were either unirradiated (Panel A) or UV irradiated with 20 J/m2 (Panel B) and reincubated at 37ºC for 6 hours before processing. 5 X 10^4 cells were placed on glass slides using a cytospin adaptor. After fixing in 100% methanol, the cells were hybridized with rabbit anti-DDX6 (MBL) and detected with a rabbit secondary antibody that was conjugated with Texas Red. The cells were then hybridized with a mouse anti-SPT5 (BD Biosciences) and detected with a mouse secondary Alexa Fluor 488 (green). The photomicroscopic images were taken at 63X with a Leica confocal microscope.

Since cytoplasmic P-bodies could be readily visualized with anti-DDX6 antibody, we examined untreated and ionizing radiation exposed (IR; 5 Gy) MCF7 cells that were concomitantly stained with both DDX6 and BRCA1 specific antisera. Visualization was

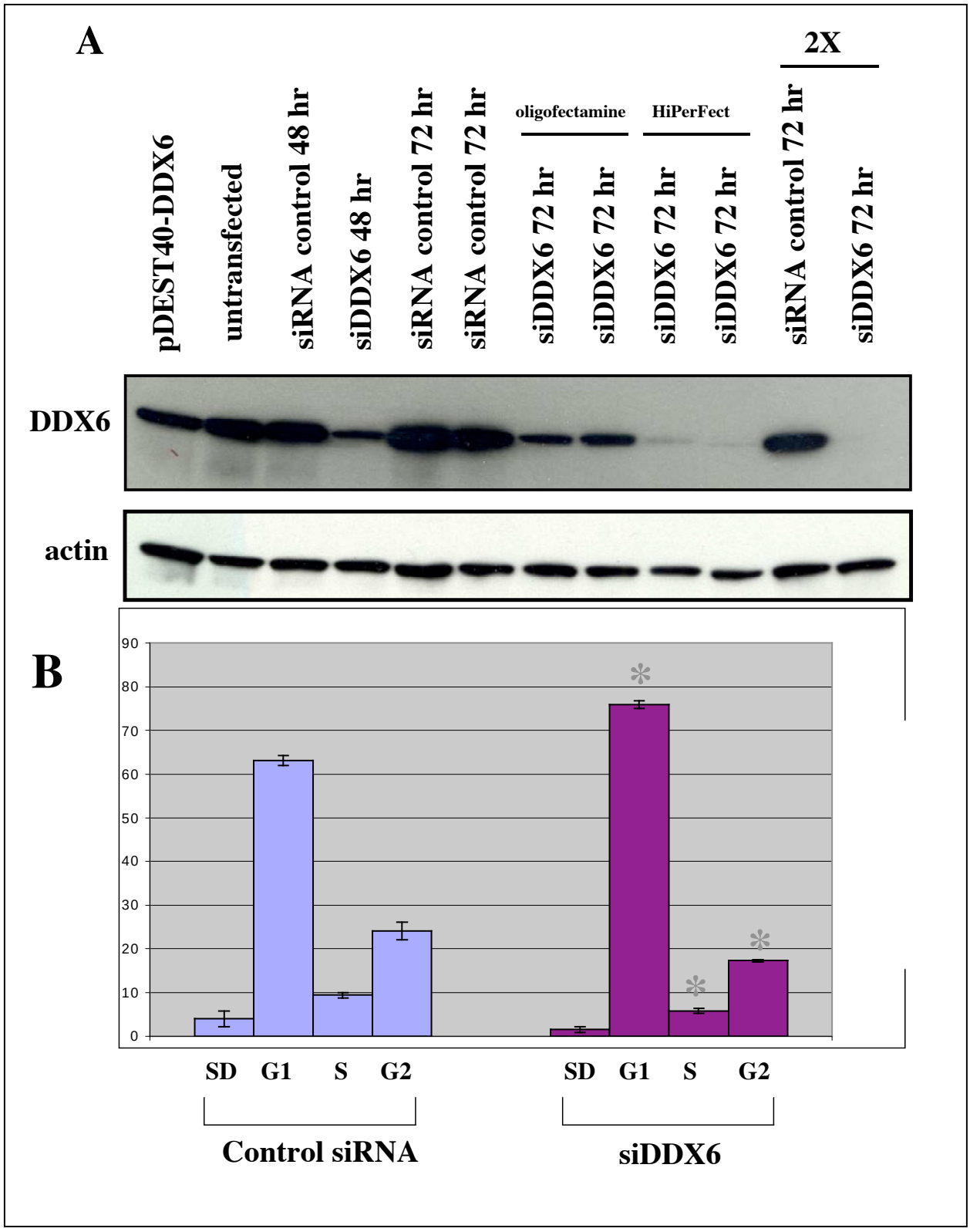

Fig. 9 Panel A. Knockdown of DDX6 protein expression by siRNA in MCF7 cells. MCF7 cells were seeded at 8 X 10^5 per 60 mm dish and transfected with siRNA directed against DDX6 utilizing either Oligofectamine (Invitrogen) or HiPerFect (Qiagen) according to manufacturers instructions. Whole cell lysates were prepared as previously described in Figure 2. Proteins were separated and visualized using the appropriate antibodies as probes using standard Western blot analysis as previously described. Successful knockdown of DDX6 was observed at 72 hours post transfection using the HiPerFect transfection reagent. Oligofectamine was ineffective for DDX6 ablation. In one experiment siRNA was transfected twice at 24 hour intervals (2x). Actin has been visualized to serve as a loading control.
Panel B. Knockdown of DDX6 results in a G1 arrest in MCF7 cells. 72 hours following transfection of DDX6 specific siRNA, cells were collected for flow cytometry. DNA content was determined using propidium iodide (Sigma). The S phase fraction of cells decreased at 72 hours in the siRNA of DDX6 as compared to the control transfection and to MCF 7 cells that were untransfected. Error bars are $\pm$ 1 standard deviation about the mean. The asterix indicate significant differences between the control and siRNA exposed cell populations.

with the appropriate fluorescent Texas Red and Alexa Fluor488 conjugated secondary antisera. As described above, the DDX6 was localized almost exclusively to the cytoplasm in the unirradiated MCF7 cells (Fig 7A). BRCA1 was diffusely stained and confined primarily to the nucleus such that widely scattered speckling was visible. Some BRCA1 staining was also evident in the cytoplasm. Upon merging the two fluorescence signals, little or no overlap (yellow) could be observed between DDX6 and BRCA1 in unirradiated MCF7 cells. Following IR, the staining pattern and distribution of DDX6 appeared unaffected as compared to unirradiated cells with DDX6 localizing primarily to discrete cytoplasmic P-body structures (Fig. 7B). BRCA1 on the other hand became more diffuse throughout the nucleus and cytoplasm (Fig. 7B). Upon merging the two fluorescent signals, clear overlap between BRCA1 and DDX6 antibodies could be visualized within the cytoplasm (Figs, 7B and 7C). These results indicate that following DNA damage, BRCA1 is shuttled from the nucleus to the cytoplasmic P-bodies.

Using the same indirect immunofluorescent staining approach, we examined whether Spt5p co-localized with DDX6 following UV damage in MCF7 cells. Unirradiated and UV irradiated (20 J/m2) MCF7 cells were fixed and stained as described above. Following staining of the unirradiated MCF7 cells, the DDX6 was visualized to localize exclusively in the cytoplasm in discrete P-body structures as described above (Fig. 8A). The Spt5 was localized primarily to the nucleus in mottled patches with a few discrete foci. Merging of the two signals failed to reveal any significant overlap between the two proteins (Fig 8A). However, following UV irradiation, Spt5 formed more numerous and intense intranuclear foci and upon merging of the two fluorescent signals, significant overlap between DDX6 and Spt5p could be observed in the cytoplasm of multiple MCF7 cells (Fig. 8B). These results clearly indicate that following DNA damage, the transcriptional elongator DSIF is translocated from the nucleus to cytoplasmic P-bodies in a manner similar to that of BRCA1. These results are in clear support of the model presented above for BRCA1 interactions with RNAPII at elongating transcription complexes whose progression has been stalled by DNA damage. Moreover, it suggest a molecular function for BRCA1 to shuttle out of the nucleus following DNA damage within actively transcribing genes.

Dhh1p exhibits the characteristics of a checkpoint adaptation protein that is specifically required for cells to reenter the cell cycle following G1 checkpoint arrest [38 47]. Thus in the absence of *DHH1*, cells undergo a prolonged G1 arrest in the presence of

DNA damage. Furthermore, *dhh1Δ* yeast cells appear to be larger in size when compared to WT which is characteristic of genes that regulate critical processes required for the G1 to S phase cell cycle transition [48]. We therefore examined the cell cycle characteristics of MCF7 cells in which the ortholog of Dhh1p (Ddx6p) had been ablated by RNA interference to determine whether Ddx6p regulated G1 to S phase transition similar to that described for Dhh1p in yeast. Using short interfering RNA substrates (siRNA) we were able to successfully knockdown DDX6 expression such that little or no Ddx6p was detectable 72 hours following transfection of the siRNA substrates (Fig. 9A). Using flow cytometry we examined the distribution of G1, S G2 and M phase MCF7 cells that were treated with control siRNA and still expressed Ddx6p versus cells in which Ddx6p had been ablated by siRNA knockdown. Similar to the effects of Dhh1p in yeast, ablation of Ddx6p in undamaged MCF7 cells resulted in a significant enhancement of the fraction of cells arrested in G1 (Fig. 9B). Moreover, as with *dhh1Δ* yeast cells, IR exposure of MCF7 cells in which Ddx6p has been ablated by siRNA, further increases the percentage of cycling cells that are arrested in the G1 phase of the cell cycle (data not shown). These results suggest that similar to Dhh1p, Ddx6p may serve as a checkpoint adaptation protein to regulate reentry into the cell cycle following DNA damage induced G1 arrest.

Key Research Accomplishments:

- Confirmation that yeast Spt4p physically interacts with BRCA1 in yeast using co-immunoprecipitation techniques.
- Optimizing the reaction conditions to promote physical BRCA1 interactions with transcriptional cofactors. Optimal NP40 concentration in extraction buffer to maintain complex interactions was found to be 0.8%. Preclearing of whole cell lysates with normal immunoglobulin prior to co-immunoprecipitation prevents non-specifc BRCA1 co-IP interaction with normal mouse or rabbit IgG.
- Identification of the RNAPII CTD specific kinase CTK1 as a major suppressor of BRCA1 induced lethality. This implicates RNAPII as the major lethal interactive target of BRCA1 in yeast.
- Identification of DNA damage dependent interactions of BRCA1 with Ddx6p and Spt5p in human MCF7 cells. This validates the yeast genetic system as a rapid method for identifying conserved cancer gene targets.
- Determination that physical interaction of Ddx6p with BRCA1 in human cells requires a functional BRCT domain within BRCA1.
- Discovery that BRCA1 expression in yeast results in cleavage of the phosphorylated form of the RNAPII C terminal domain (CTD). Two separate cancer related mutations within the BRCT domain of BRCA1 (a substitution mutation and a terminal 10 amino acid truncation) fail to cleave the RNAPII CTD following overexpression confirmed by Western analysis.
- Expression of full length BRCA1 fails to cleave the RNAPII CTD in *ccr4Δ, dhh1Δ* and *def1Δ* suppressor of BRCA1 induced lethality.
- BRCA1 and Spt5p but not Ddx6p were found to be rapidly degraded following exposure of human MCF7 cells to the DNA damaging agent MMS.

This indicates that Ddx6p function may be quite different in the suppression of BRCA1 induced lethality in yeast

- Discovery that BRCA1 and Spt5p colocalize at cytoplasmic processin (P)-bodies. Since these are sites of mRNA degradation, this suggests that BRCA1 interacts with elongating transcription complexes blocked by DNA damage and "escorts" the mRNA RNAPII complexes into the cytoplasmic P-bodies to enable degradation of prematurely terminated mRNA.
- Using siRNA technology, we have successfully ablated Ddx6p in MCF7 cells Following ablation, the fraction (percent) of MCF7 cells in G1 increases. Ionizing radiation of Ddx6p ablated MCF7 cells further increases the percentage of cells in G1.

Reportable Outcomes

Attended and presented at two meetings::

GSA meeting: Genetic Analysis: Model Organisms to Human Biology Jan. 5-7 San Diego, California

Disease Models for Target Discovery and Target Validation
Oct. 18-19, World Trade Center, Boston MA

Abstracts for these meetings are located in the Appendix.

One paper has been submitted and is being revised. Two other manuscripts based on the work presented here are in preparation.

Conclusions:

Our research using the yeast *Saccharomyces cerevisiae* has allowed us to gain significant new insight into the molecular functions of the breast cancer suppressor BRCA1. This project has used the yeast as a model system to identify new genetic and physical targets that physically interact with BRCA1 in yeast and human cells. From the identification of these targets we appear to have elucidated the molecular mechanism of BRCA1 induced lethality in yeast. Moreover, we have been able to develop a molecular model that accounts for the shuttling function of BRCA1 from the nucleus into the cytoplasm of human cells. Our model states that following DNA damage in actively transcribing genes, RNAPII elongation is arrested by the blockage to polymerase progression. In conjunction with a variety of cofactors including the DSIF complex (Spt4-Spt5), BRCA1 binds to the phosphorylated form of the RNAPII CTD. This promotes cleavage of the CTD and release of the arrested transcription complex from the damaged DNA template. Subsequently, repair enzymes gain access to the damage site to initiate DNA repair. BRCA1 "escorts" the RNAPII complex and the prematurely terminated mRNA to the cytoplasmic P-body where mRNA degradation is initiated. Until the mRNA is degraded, a damage signal persists to prolong G1 arrest and prevent cell cycle progression until the DNA repair has been finalized. Thus BRCA1 is proposed

to plays a critical role in signaling the presence of DNA damage within actively transcribing genes. Furthermore, BRCA1 appears to play a key role in repair by disassembling elongating transcription complexes whose progression has been blocked by DNA damage. Given the highly conserved nature of the proteins involved in this process, this repair pathway may play a key role in tumor suppression in breast and ovarian cancer

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APPENDIX

PRESENTED AT: DISEASE MODELS FOR TARGET DISCOVERY AND TARGET VALIDATION
OCT. 18-19, WORLD TRADE CENTER, BOSTON MA

IDENTIFICATION OF A HIGHLY CONSERVED BREAST CANCER GENE NETWORK USING YEAST AS A MODEL ORGANISM

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We have used the genetic accessibility of the isogenic ***Saccharomyces cerevisiae*** deletion collections to identify a highly conserved network of gene targets that interact with the tumor suppressor protein BRCA1. This breast cancer gene has been implicated in a wide variety of DNA metabolic functions that maintain genome integrity including DNA damage signaling, checkpoint activation, transcription, centrosome duplication and recombination. To elucidate a fundamental new role for BRCA1, we selected diploid *Saccharomyces.cerevisiae* deletion mutants that suppressed the G1 arrest and lethality observed following heterologous expression of BRCA1. A high copy, selectable (*URA3*) plasmid containing BRCA1 fused to the galactose promoter was transformed into a pool of 4746 diploid deletion strains each carrying a unique 20 bp molecular tag. Transformed cells were immediately plated to synthetic complete galactose - uracil medium (BRCA1 expressed) and rapidly growing mutant colonies were isolated. Deletion strains were identified by sequencing the unique 20 bp tag and BRCA1 expression was confirmed by immunofluorescence or Western blot analysis. We also screened our collection of ionizing radiation (IR) sensitive diploid deletion strains for those that could suppress the BRCA1-induced G1 arrest and lethality. From these screens we independently identified highly conserved interactive components of the CCR4 damage response network as well as factors that appear to participate in transcription elongation. The majority of these genes, including *CCR4, DHH1, DEF1, HCM1, SPT4, SPT5, SUB1, YAF9* and numerous components of the nuclear pore complex are required for transcription and confer resistance to IR as well as the transcription elongation inhibitors mycophenolic acid (MPA) and/or zymocin. Furthermore, when these genes are deleted, they mediate a prolonged G1 cell cycle arrest following DNA damage but conversely allow rapid G1/S cell cycle transition following BRCA1 expression. Moreover, nonlethal doses of transcription elongation inhibitors greatly augmented BRCA1-induced lethality in WT yeast. We propose that in WT yeast, BRCA1 binds to elongating transcription complexes and stalls mRNA elongation in G1. Alternatively, transcription elongation may stall when DNA damage is encountered. Eventually, cells adapt to this persistent DNA damage and enter S phase where the stalled transcription complexes serve as replication blocks that are processed into lethal DNA double-strand breaks. Consistent with this idea is the observation that BRCA1 induces significantly enhanced plasmid degradation and loss in WT as compared to *SPT4* deleted cells and much of the BRCA1 protein expressed in yeast is truncated from the C terminal end by a process that does not involve protein degradation. Using co-immunoprecipitation, we determined that Ccr4p, Dhh1p and Spt4p physically interact with BRCA1 in yeast, while the highly conserved human ortholog of Dhh1p (DDX6) physically interacts with BRCA1 in human cells. The formation of immunofluorescent BRCA1 foci following treatment of human MCF7 cells with MPA strongly suggests that BRCA1 similarly

interacts with stalled elongating transcription complexes at sites of DNA damage in human cells. This suggests that inhibition of transcription elongation may be of therapeutic value in the treatment of breast cancer disease. Thus we have successfully used yeast as a functional genomic "tool" for the rapid identification of a novel, highly conserved mRNA damage surveillance pathway in which BRCA1 plays a critical role. Moreover, similar success in the identification of novel therapeutic gene targets could be realized following the expression of any human disease gene in this model organism.

APPENDIX

Presented at the Genetics Society of America meeting: Genetic Analysis: Model Organisms to Human Biology Jan. 5-7 San Diego, California

BRCA1 interacts with conserved components of the transcription elongation complex

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To elucidate a conserved role for the tumor suppressor BRCA1, we selected *S. cerevisiae* mutants that suppressed the G1 arrest and lethality normally observed following expression of BRCA1 in yeast. A high copy, selectable plasmid containing BRCA1 fused to the GAL1 promoter was transformed into a pool of 4746 deletion strains each carrying a unique 20bp sequence tag. Transformed cells were plated to GAL to express BRCA1 and rapidly growing colonies were identified by tag sequencing. A collection of ionizing radiation (IR) sensitive deletion strains was similarly screened for BRCA1 suppressors. Together, these screens identified highly conserved interactive components of the CCR4 damage response network as well as factors that participate in transcription elongation. The majority of these genes, including CCR4, DHH1, DEF1, SPT4, SPT5, SUB1, YAF9, HCM1 and numerous components of the nuclear pore complex confer resistance to IR as well as the transcription elongation inhibitors mycophenolic acid (MPA) and zymocin. When deleted these genes mediate a prolonged G1 cell cycle arrest following DNA damage but conversely allow rapid G1/S cell cycle transition following BRCA1 expression. Furthermore, BRCA1 expression enhanced degradation and loss of actively transcribing plasmids in WT as compared to *spt4* cells. Using co-immunoprecipitation, we determined that Spt4p, Ccr4p and Dhh1p physically interact with BRCA1 in yeast, while the human ortholog of Dhh1p (DDX6) interacts with BRCA1 in human cells. Thus BRCA1 appears to destabilize the genome by binding to transcription complexes that stall mRNA elongation in G1. Similarly, transcription may stall at DNA damage sites. As cells enter S phase the stalled transcription complexes serve as replication blocks that are processed into lethal DNA double-strand breaks. The formation of BRCA1 foci following exposure of MCF7 cells to MPA suggests that BRCA1 similarly interacts with stalled transcription complexes at sites of DNA damage in human cells. Thus yeast can serve as a functional genomic tool for the identification of new cancer gene networks.